HPLC and UHPLC for Practicing Scientists
2nd Edition

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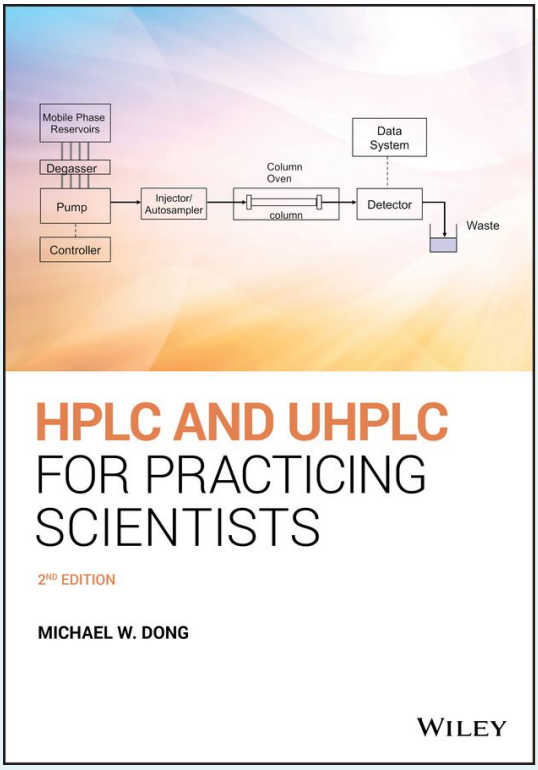

1

INTRODUCTION

1.1 INTRODUCTION

1.1.1 Scope

High-performance liquid chromatography (HPLC) is a versatile analytical (separation) technique widely used for the analysis of pharmaceuticals, biomolecules, polymers, and many organic and ionic compounds. There is no shortage of excellent books on chromatography [1–3] and on HPLC [4–11], though many are outdated and others tend to focus more on academic theories or specialized topics. This book strives to be a concise and an all-inclusive text that "capsulizes" the essence of HPLC fundamentals, applications, and developments. It describes the fundamental theories and terminologies for the novice and reviews relevant concepts, best practices, and modern trends for the experienced practitioner. While broad in scope, this book focuses on reversed-phase HPLC (the most common separation mode) and pharmaceutical applications (the most significant user segment). Information is presented straightforwardly and is illustrated with an abundance of figures, chromatograms, tables, and case studies, supported by selected key references or web resources.

Most importantly, this book was written as an updated reference guide for busy laboratory analysts and researchers. Topics covered include HPLC operation, method development, maintenance/troubleshooting, and the regulatory aspects of pharmaceutical analysis. This book can serve as a supplementary text for students pursuing a career in analytical chemistry and pharmaceutical science. A reader with a science degree and a basic understanding of chemistry is assumed. This second edition continues the same theme as the first edition [4] with updates on all chapters plus three new chapters on ultra-high-pressure liquid chromatography (UHPLC), liquid chromatography–mass spectrometry (LC/MS), and the analysis of recombinant biologics (biopharmaceuticals). A quiz section at the end of each chapter serves as a teaching/evaluation aid.

This book offers the following benefits:

- A broad-scope overview of fundamental principles, instrumentation, columns, and applications.

HPLC and UHPLC for Practicing Scientists, Second Edition. Michael W. Dong.

- A concise review of concepts and trends of modern HPLC.
- An update of best practices in HPLC operation, method development, maintenance, troubleshooting, and regulatory aspects in analytical testing.
- New standalone overview chapters on UHPLC, LC/MS, and analysis of recombinant biologics.

1.1.2 What Is HPLC?

Liquid chromatography (LC) is a physical separation technique conducted between two phases – a solid phase and a liquid phase. A sample is separated into its constituent components (or analytes) by distributing (via partitioning, adsorption, or other interactions) between the mobile phase (a flowing liquid) and a solid stationary phase (sorbents packed inside a column). For example, the flowing liquid can be an organic solvent such as hexane and the stationary phase can be the porous silica particles packed into a column. HPLC is a modern form of LC that uses small-particle columns through which the mobile phase is pumped at high pressure.

Figure 1.1a is a schematic of the chromatographic process, where a mixture of components A and B are separated into two distinct bands as they migrate down the column filled with packing (stationary phase). Figure 1.1b is a microscopic representation of the dynamic partitioning process of the analytes between the flowing liquid and the stationary phase attached to a spherical packing particle. Note that the movement of component B is retarded in the column because each B molecule has a stronger affinity for the stationary phase than the A

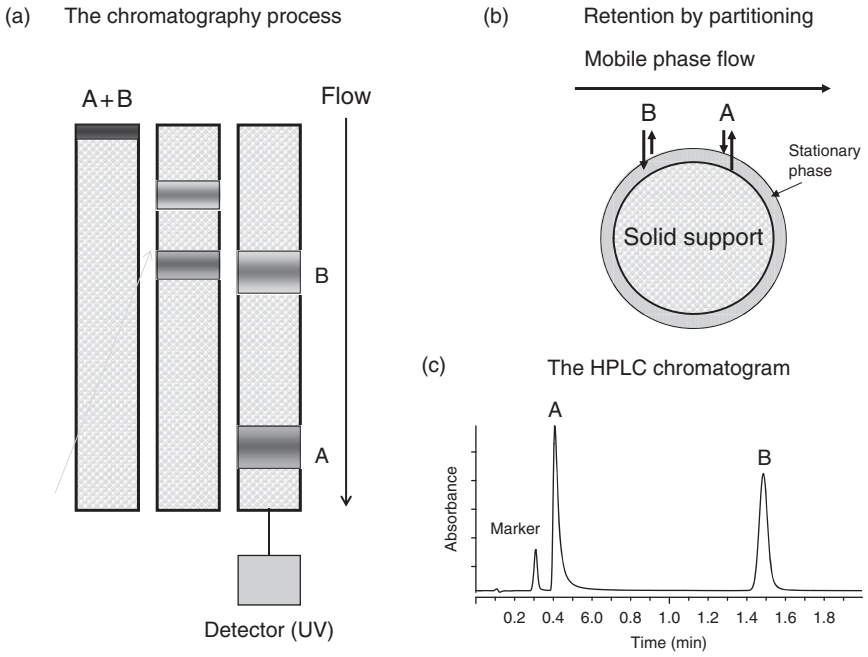

Figure 1.1. (a) Schematic of the chromatographic process showing the migration of two bands of components down a column. (b) Microscopic representation of the partitioning process of analyte molecules A and B into the stationary phase bonded to a solid spherical support. (c) A chromatogram plotting the signal from a UV detector displays the elution of components A and B.

molecule. An inline detector monitors the concentration of each separated component band in the effluent and generates a signal trace called the "chromatogram," shown in Figure 1.1c.

1.1.3 A Brief History

The term *chromatography* meaning "color writing" was first used by Mikhail Tsvet, a Russian botanist who separated plant pigments on chalk ($CaCO_3$) packed in glass columns in 1903. Since the 1930s, chemists have used gravity-fed silica columns to purify organic materials and ion-exchange resin columns to separate ionic compounds and radionuclides. The invention of gas chromatography (GC) by the British biochemists A. J. P. Martin and R. L. M. Synge in 1952 and its successful applications provided the theoretical foundation and the incentive for the development of LC. In the late 1960s, LC turned "highperformance" with the use of small-particle columns that required high-pressure pumps. The first generation of HPLCs was developed by researchers in the 1960s, including Joseph Huber in Europe and Csaba Horváth and Jack Kirkland in the United States. Commercial development of inline detectors and reliable injectors allowed HPLC to become a sensitive and quantitative technique leading to an explosive growth of applications [4, 5]. In the 1980s, the versatility and precision of HPLC rendered it virtually indispensable in pharmaceutical and many diverse industries. The annual worldwide sales of HPLC systems and accessories were about four billion US$ in 2016 [12]. (http://www.marketsandmarkets.com/PressReleases/chromatography-instrumentation.asp) Today, HPLC continues to evolve rapidly toward higher speed, efficiency, and sensitivity, driven by the emerging needs of life sciences and pharmaceutical applications. Figure 1.2a depicts the classical technique of LC with a glass column packed with coarse adsorbents and gravity fed with solvents. Fractions of the eluent containing separated components are collected manually and subsequently analyzed by spectrometry. This low-pressure LC is contrasted with the latest computer-controlled

(a) (b)

Figure 1.2. (a) The traditional technique of low-pressure liquid chromatography using a glass column and gravity-fed solvent with manual fraction collection. (b) A modern automated UHPLC instrument (Waters Acquity UPLC system) capable of very high efficiency and pressure up to 15 000 psi.

Table 1.1. Advantages and Limitations of HPLC

Advantages
• Applicable to diverse analyte types
• Precise and highly reproducible quantitative analysis
• HPLC coupled with mass spectrometry (HPLC/MS)
• High separation power with sensitive detection
Perceived limitations
• Lack of an ideal universal detector
• Less separation efficiency than capillary gas chromatography (GC)
• Still arduous for regulatory or quality control (QC) testing

UHPLC, depicted in Figure 1.2b, operated at very high pressures, and capable of exceptional high separation efficiency.

1.1.4 Advantages and Limitations

Table 1.1 highlights the advantages and limitations of HPLC. HPLC is a premier separation technique capable of multicomponent analysis of complex mixtures. Few analytical techniques can match its versatility and precision of <0.1–0.5% relative standard deviation (RSD). HPLC can be highly automated, using sophisticated autosamplers and data systems for unattended analysis and report generation. A host of highly sensitive and specific detectors extend detection limits to nanogram, picogram, and even femtogram levels. As a preparative technique, it provides quantitative recovery of many labile components in milligram to kilogram quantities. Most importantly, HPLC is amenable to 60–80% of all existing compounds, as compared with about 15% for gas chromatography (GC) [3, 4].

Historically, HPLC is known to have several disadvantages or perceived limitations. First, there is no universal detector, such as the equivalence of a thermal conductivity or flame ionization detector in GC. So detection is more problematic if the analyte does not absorb UV radiation or is not ionized for mass spectrometric detection. Second, separation efficiency is less than that of capillary column GC. Thus, the analysis of complex mixtures is more difficult. Finally, HPLC has many operating parameters and can be difficult for a novice to develop new methods. As shown in later chapters, these limitations have been minimized mainly through the recent instrument and column developments.

1.1.5 Ultra-High-Pressure Liquid Chromatography (UHPLC)

UHPLC is the latest and the most important development in HPLC. It uses equipment with very high pressures together with columns packed with small particles and is capable of facilitating faster separations with high efficiency. UHPLC shares most of theories and applications with HPLC, which are discussed in the various chapters of this book. The history, benefits, best practices, and potential issues of UHPLC are described as a standalone topic in Chapter 5.

1.2 PRIMARY MODES OF HPLC

In this section, the four primary separation modes of HPLC are introduced and illustrated with application examples, labeled with the pertinent parameters: column (stationary phase),

mobile phase, flow rate, detector, and sample information. These terminologies will be elaborated in later chapters.

1.2.1 Normal-Phase Chromatography (NPC)

Also known as liquid–solid chromatography or adsorption chromatography, normal-phase chromatography (NPC) is the traditional separation mode based on adsorption/desorption of the analyte onto a polar stationary phase (typically silica or alumina) [3–5]. Figure 1.3a shows a schematic diagram of a porous silica particle with silanol groups (Si-OH) residing at the surface and inside its pores. Polar analytes migrate slowly through the column due to strong interactions with the silanol groups. Figure 1.4 shows a chromatogram of four vitamin E isomers in a palm olein sample using a nonpolar mobile phase of hexane modified with ethanol. It is believed that a surface layer of water reduces the activity of the silanol groups and allows for more symmetrical peaks [3]. NPC is particularly useful for the separation of nonpolar compounds and isomers, as well as for the fractionation of complex samples by functional groups or sample clean-up. One significant disadvantage of this mode is the easy contamination of the polar surfaces by highly retained sample components rendering it a less reproducible technique. This problem is reduced by bonding polar functional groups such as amino- or cyano-moiety to the silanol groups. Today, NPC is primarily used in chiral separations and preparative applications.

1.2.2 Reversed-Phase Chromatography (RPC)

The separation is based on analytes' partition coefficients between a polar mobile phase and a hydrophobic (nonpolar) stationary phase. The earliest stationary phases were solid particles coated with nonpolar liquids. These were quickly replaced by permanently covalently bonded hydrophobic groups, such as octadecyl (C18) bonded groups on a silica support. A simplified schematic view of reversed-phase chromatography (RPC) is shown in Figure 1.3b, where

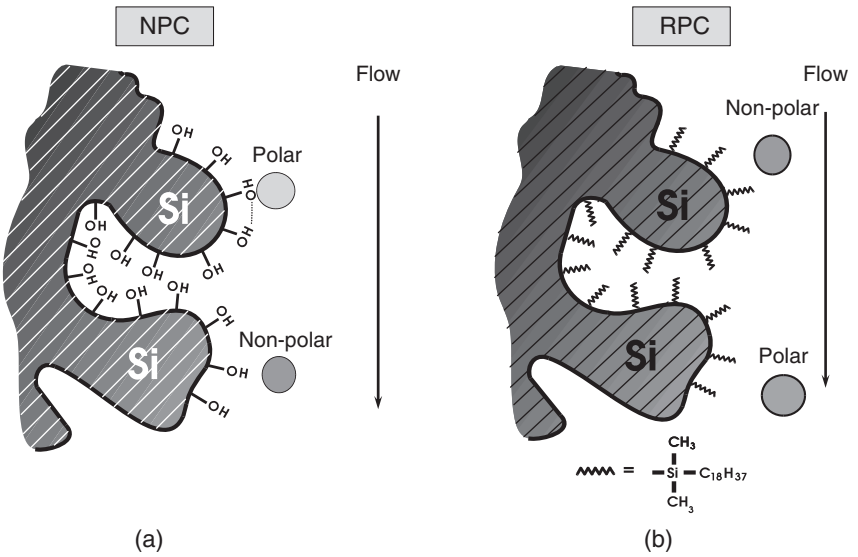

Figure 1.3. Schematic diagrams depicting separation modes of (a) normal-phase chromatography (NPC) and (b) reversed-phase chromatography (RPC).

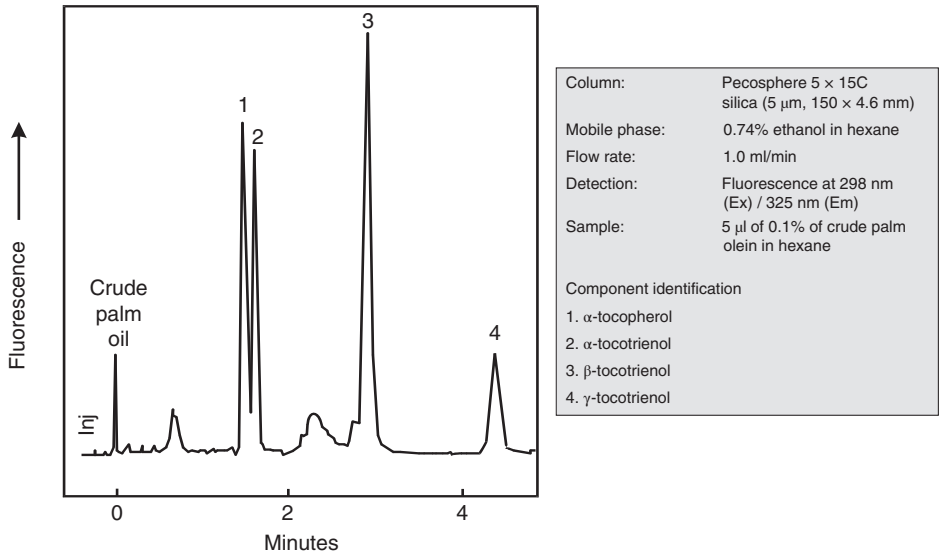

Figure 1.4. A normal-phase HPLC chromatogram of a palm olein sample showing the separation of various isomers of vitamin E. Source: Courtesy of PerkinElmer.

polar analytes elute first while nonpolar analytes elute later by interacting more strongly with the hydrophobic C18 groups that form a "liquid-like" layer around the solid silica support. This elution order of "polar first and nonpolar last" is the reverse order of that observed in NPC, and thus the term "reversed-phase chromatography." RPC typically uses a mixture of methanol or acetonitrile with water. The mechanism of separation is mainly attributed to hydrophobic or "solvophobic" interaction [13, 14]. The term "solvophobic interaction" refers to the relatively strong cohesive forces between the polar solvent molecules themselves and with the hydrated analytes and their interaction with the nonpolar stationary phase. Figure 1.5 shows the separation of three organic components. Note that uracil, the most polar component and a highly water-soluble compound, elutes first. *t*-Butylbenzene elutes much later due to increased hydrophobic interaction with the stationary phase. RPC is the most popular HPLC mode and is used in more than 70% of all HPLC analyses [3, 4]. It is suitable for the analysis of polar (water-soluble), medium-polarity, and some nonpolar analytes. Ionic analytes can be separated using ion-suppression or ion-pairing techniques discussed in Chapter 2. RPC is used extensively in purity analysis or stability-indicating assays because the weak dispersive forces responsible for solute retention give assurance that all sample components are eluted from the column.

1.2.3 Ion-Exchange Chromatography (IEC)

In ion-exchange chromatography (IEC) [3–5], the separation mode is based on the exchange of ionic analytes with the counter ions of the ionic groups attached to the solid support (Figure 1.6a). Typical stationary phases are a cationic exchange (sulfonate) or anionic exchange (quaternary ammonium) groups bonded to polymeric or silica materials. Mobile phases consist of buffers, often with increasing ionic strength (e.g. a higher concentration of NaCl), to force the migration of the analytes. Common applications are the analysis of ions and biological components such as amino acids, proteins/peptides, and polynucleotides.

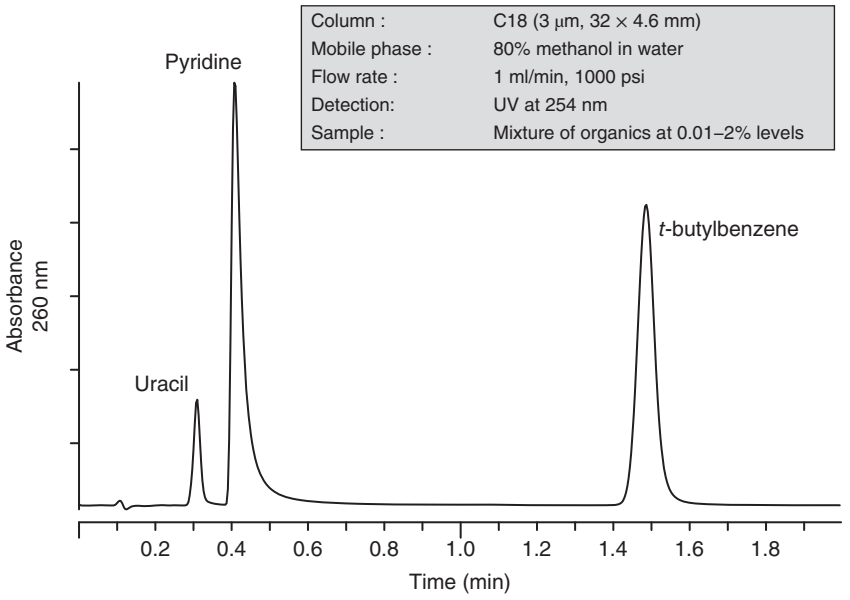

Figure 1.5. A reversed-phase HPLC chromatogram of three organic components eluting in the order of "polar first and nonpolar last." The basic pyridine peak is tailing due to a secondary interaction of the nitrogen lone pair with residual silanol groups of the silica-based bonded phase. Source: Ahuja and Dong 2005 [9]. Copyright 2005. Reprinted with permission of Elsevier.

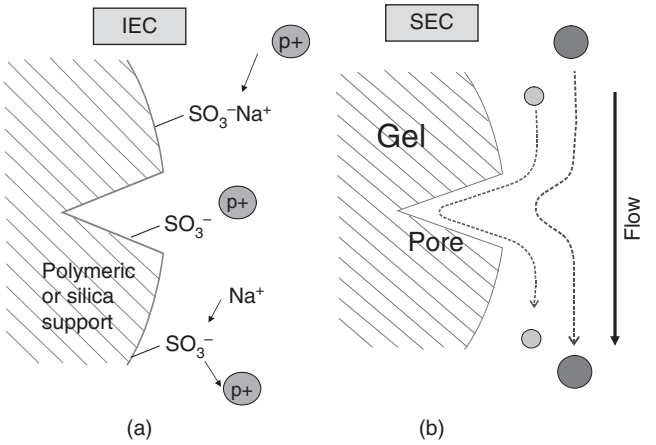

Figure 1.6. (a) Schematic diagrams depicting separation modes of (a) ion-exchange chromatography (IEC), showing the exchange of analyte ion p+ with the sodium counter ions of the bonded sulfonate groups. (b) Size-exclusion chromatography (SEC), showing the faster migration of large molecules.

Figure 1.7 shows the separation of amino acids on a sulfonated polymer column and a mobile phase of increasing sodium ion concentration and increasing pH. Since amino acids do not absorb strongly in the UV or visible region, a postcolumn reaction technique is used to form colored derivatives to allow sensitive detection at 550 nm. Ion chromatography [15] is a segment of IEC pertaining to the analysis of cations or anions using a high-performance ion-exchange column, usually with a specialized suppressed conductivity detector.

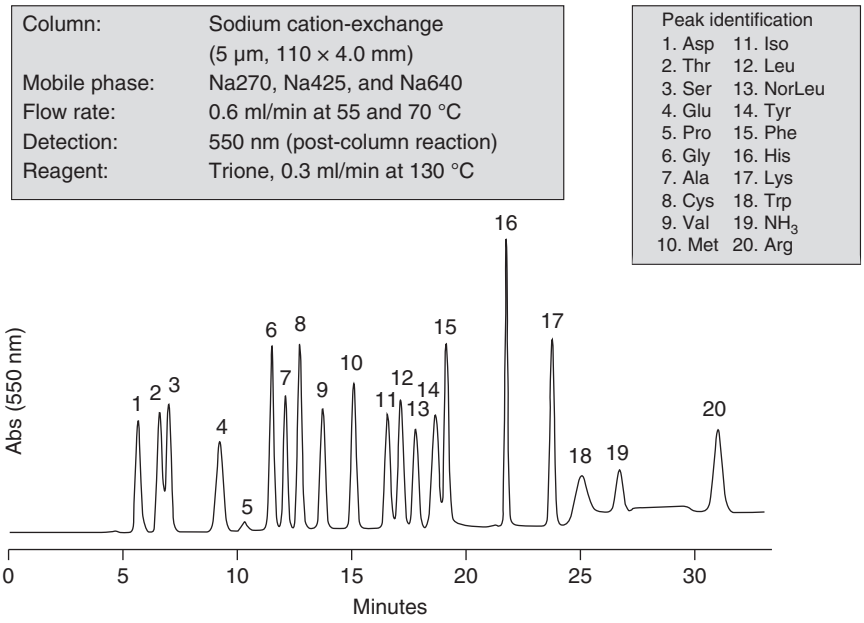

Figure 1.7. An IEC chromatogram of essential amino acids using a cationic sulfonate column and detection with postcolumn reaction. Note that Na270, Na425, and Na640 are prepackaged eluents containing sodium ions (NaCl) and buffered at pH of 2.70, 4.25, and 6.40, respectively. Trione is a derivatization reagent similar to ninhydrin used for postcolumn derivatization. Source: Courtesy of Pickering Laboratories.

1.2.4 Size-Exclusion Chromatography (SEC)

Size-exclusion chromatography (SEC) [16] is a separation mode based solely on the analyte's molecular size. Figure 1.6b shows that a large molecule is excluded from the pores and migrates quickly, whereas a small molecule can penetrate all the inner pores and migrates more slowly down the column. It is often called gel permeation chromatography (GPC) when used for the determination of molecular weights of organic polymers and gel-filtration chromatography (GFC) when used in the separation of water-soluble biological compounds. In GPC, the column is packed with cross-linked polystyrene beads of controlled pore sizes and eluted with mobile phases such as toluene or tetrahydrofuran. Figure 1.8 shows the separation of polystyrene standards showing an elution order of decreasing molecular size. Detection with a refractive index or UV detector is typical. SEC is a low-resolution technique in which interaction of the solute with the stationary phase (support) besides size exclusion should be avoided.

1.2.5 Other Separation Modes

Besides the four primary HPLC separation modes, several other modes or related techniques are noted below.

- *Affinity chromatography* [5]: Based on a receptor/ligand interaction in which immobilized ligands (enzymes, antigens, or hormones) on solid supports are used to isolate selected components from a mixture. The retained components can later be released in as a purified fraction.

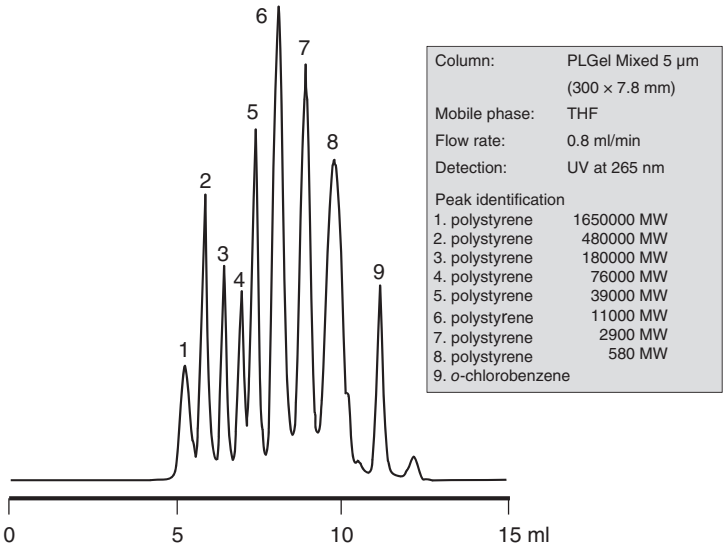

Figure 1.8. A GPC chromatogram of polystyrene standards on a mixed-bed polystyrene column. Source: Courtesy of Polymer Laboratories (Agilent Technologies).

- *Chiral chromatography* [17]: For the separation of enantiomers using a chiral-specific stationary phase. Both NPC and RPC chiral columns are available.
- *Hydrophilic interaction chromatography (HILIC)*: This is somewhat similar to NPC using a polar stationary phase such as silica or ion-exchange materials but eluted with polar mobile phases of organic solvents and aqueous buffers. It is commonly used to separate polar analytes, ions, hydrophilic peptides, glycans, and polar metabolites.
- *Hydrophobic interaction chromatography (HIC)*: Analogous to RPC using the same type of stationary phases except that it uses mobile phases of low organic solvent content and high salt concentrations. It is commonly used for the separation of proteins that are readily denatured by RPC mobile phases. HIC is particularly useful in the analysis of antibody–drug conjugates (ADCs).
- *Electrochromatography*: Uses capillary electrophoresis [18] (CE) equipment with a packed capillary column. The mobile phase is driven by the electromotive force from a high-voltage source as opposed to a mechanical pump. It is capable of very high efficiencies.
- *Supercritical fluid chromatography (SFC)* [19]: Uses HPLC-type packed columns and a mobile phase of pressurized supercritical fluids (i.e. carbon dioxide modified with a polar organic solvent). It is useful for nonpolar analytes and preparative applications where purified materials can be recovered quickly by evaporating the carbon dioxide. HPLC pumps with cryogenic cooling are often used with UV and MS detectors. There was a recent resurgence of interest in SFC brought forth by newer instruments of improved precision, sensitivity, and reliability. SFC is becoming the preferred technique in chiral separation and high-throughput purification in drug discovery research.
- *Other forms of low-pressure liquid chromatography*:
  - Thin-layer chromatography (TLC) [20] uses glass plates coated with adsorbents and capillary action as the driving force. Useful for sample screening and semiquantitative analysis.

— Paper chromatography (PC), a form of partition chromatography using paper as the stationary phase and capillary action as the driving force.
— Flash chromatography, a semipreparative technique for sample purification using disposable glass NPC columns and mobile phases driven by gas-pressure or low-pressure pumps.

1.3 SOME COMMON-SENSE COROLLARIES

The goal of most HPLC analysis is to separate analyte(s) from other components in the sample for accurate quantitation. Several common corollaries are often overlooked by practitioners:

1. *HPLC is not complicated but complex*: It does not require an advanced scientific degree to understand its core concepts and/or excel in its practices. It is, however, complex due to the scope of equipment components and operating variables (i.e. column, mobile phases, pump, autosamplers, detectors, data systems, operating conditions, samples, standards, diluent) working in tandem to generate robust and accurate results.
2. *The sample must be soluble:* "*If it's not in solution, it cannot be analyzed by HPLC*." Solubility issues often complicate assays of low-solubility analytes or components, which are difficult to be extracted from sample matrices. Low recoveries usually stem from poor sample preparation steps rather than the HPLC analysis itself.
3. *For separation to occur, analytes must be retained and have differential migration in the column*: Separation cannot happen without retention and sufficient differential interaction with the stationary phase. For quantitative analysis with UV detection, analytes must have different retention on the column vs. other components. Baseline resolution is usually not needed for MS detection since specific signals can be customized for each ion according to its molecular weight.
4. *The mobile phase controls the separation*: Whereas the stationary phase provides a media for analyte interaction, the mobile phase controls the overall separation. In HPLC method development, efforts focus on finding a set of mobile phase conditions with the appropriate stationary phase for separating the analyte(s) from other components. Exceptions to this rule are size exclusion, chiral, and affinity chromatography where the mobile phase plays a minor role.
5. *All C18-bonded phase columns are not the same and cannot be interchanged for critical assays*: There are hundreds of C18 columns on the market. They vary tremendously in their retention and silanol characteristics [9]. For critical assays of complex samples, C18 columns are not interchangeable and the exact column from the specific manufacturer should be used. For more straightforward potency assays of the main component, a similar C18 bonded column of identical dimension can often be substituted.
6. *The final analyte solution should be prepared in the mobile phase A*: The final analyte solution, if possible, should be dissolved in the mobile phase or a solvent of "weaker" strength (mobile phase A by convention) or the starting mobile phase in a gradient analysis. Many anomalies such as splitting or fronting peaks are caused by injecting samples dissolved in diluents stronger than the starting mobile phase. If a stronger diluent must be used to dissolve the sample, a smaller injection volume (i.e. 2–5 µl) should be considered to minimize these problems.

7. *There are no perfect methods, and every analytical method has its caveats, limitations, or pitfalls*: An experienced scientist can identify these potential pitfalls and find conditions to minimize any issues.

An article on common-sense corollaries in HPLC was published elsewhere with more extensive discussions [21].

1.4 HOW TO GET MORE INFORMATION

The reader is encouraged to obtain more information from the following sources:

- Courses sponsored by training institutions [22], manufacturers, or national meetings (American Chemical Society, Pittsburgh Conference, Eastern Analytical Symposium).
- Computer-based training programs [23].
- Useful books [4–9] and websites [24–26] of universities and other government or compendia agencies, such as the U.S. Food and Drug Administration (FDA), U.S. Environmental Protection Agency (EPA), International Conference on Harmonization (ICH), the United States Pharmacopoeia, (USP), Association of Official Analytical Chemist International (AOAC), and American Society of Testing and Materials (ASTM).
- Research and review articles published in journals [27–30] such as the *Journal of Chromatography*, *Journal of Chromatographic Science*, *Journal of Liquid Chromatography*, *LCGC Magazine*, *Analytical Chemistry*, *American Pharmaceutical Review, Journal of Separation Science*, and *American Laboratories*.

1.5 SUMMARY

This introductory chapter describes the scope of the book and gives a summary of the history, advantages, limitations, and common-sense axioms of HPLC. Primary separation modes are discussed and illustrated with examples. Information resources on HPLC are also listed.

1.6 QUIZZES

1. The most popular chromatographic mode is
(a) IEC
(b) RPC
(c) SEC
(d) NPC

2. Which molecule elutes the latest in SEC?
(a) large molecule
(b) polar
(c) nonpolar
(d) small molecule

3. Which is NOT a major advantage of HPLC?
(a) amenable to diverse sample types
(b) HPLC/MS

(c) high sensitivity
(d) simplicity for regulatory testing

4. NPC is NOT useful for
(a) protein separation
(b) SFC
(c) chiral separation
(d) purification

5. Analysis of amino acid is likely to use
(a) NPC
(b) IEC
(c) SEC
(d) RPC

1.6.1 Bonus Quiz

In your own words, describe the reasons why HPLC is the most popular analytical technique for quantitative analysis of complex samples.

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CONTENTS

Author's Biography xvii

Biographies of Contributors xix

Preface xxi

Foreword xxiii

Acknowledgments xxv

1 Introduction 1

1.1 Introduction / 1
1.1.1 Scope / 1
1.1.2 What Is HPLC? / 2
1.1.3 A Brief History / 3
1.1.4 Advantages and Limitations / 4
1.1.5 Ultra-High-Pressure Liquid Chromatography (UHPLC) / 4
1.2 Primary Modes of HPLC / 4
1.2.1 Normal-Phase Chromatography (NPC) / 5
1.2.2 Reversed-Phase Chromatography (RPC) / 5
1.2.3 Ion-Exchange Chromatography (IEC) / 6
1.2.4 Size-Exclusion Chromatography (SEC) / 8
1.2.5 Other Separation Modes / 8
1.3 Some Common-Sense Corollaries / 10
1.4 How to Get More Information / 11
1.5 Summary / 11
1.6 Quizzes / 11
1.6.1 Bonus Quiz / 12
References / 12

2 Basic Terms and Concepts 15

2.1 Scope / 15
2.2 Basic Terms and Concepts / 16
2.2.1 Retention Time (t_R), Void Time (t_M), Peak Height (h), and Peak Width (w_b) / 16
2.2.2 Retention Volume (V_R), Void Volume (V_M), and Peak Volume / 16
2.2.3 Retention Factor (k) / 18
2.2.4 Separation Factor (α) / 19

2.2.5 Column Efficiency and Plate Number (N) / 20
2.2.6 Peak Volume / 20
2.2.7 Height Equivalent to a Theoretical Plate or Plate Height (HETP or H) / 21
2.2.8 Resolution (R_s) / 21
2.2.9 Peak Symmetry: Asymmetry Factor (A_s) and Tailing Factor (T_f) / 23
2.3 Mobile Phase / 24
2.3.1 General Requirements / 24
2.3.2 Solvent Strength and Selectivity / 25
2.3.3 pH Modifiers and Buffers / 27
2.3.4 Acidic Mobile Phases / 28
2.3.5 Ion-Pairing Reagents and Chaotropic Agents / 29
2.3.6 High-pH Mobile Phases / 29
2.3.7 Other Operating Parameters: Flow Rate (F) and Column Temperature (T) / 30
2.4 The Resolution Equation / 31
2.5 The Van Deemter Equation / 33
2.6 Isocratic vs. Gradient Analysis / 35
2.6.1 Peak Capacity (n) / 35
2.6.2 Gradient Parameters (Initial and Final Solvent Strength, Gradient Time (t_G), and Flow Rate) / 36
2.6.3 The 0.25 Δt_G Rule: When Is Isocratic Analysis More Appropriate? / 37
2.7 The Concept of Orthogonality and Selectivity Tuning / 38
2.8 Sample Capacity / 41
2.9 Glossary of HPLC Terms / 41
2.10 Summary and Conclusion / 42
2.11 Quizzes / 42
2.11.1 Bonus Quiz / 44
References / 44

3 HPLC Columns and Trends **45**

3.1 Scope / 45
3.1.1 Glossary and Abbreviations / 45
3.2 General Column Description and Characteristics / 46
3.2.1 Column Hardware – Standard vs. Cartridge Format / 47
3.3 Column Type / 47
3.3.1 Types Based on Chromatographic Mode / 48
3.3.2 Column Types Based on Dimension / 48
3.3.3 Column Length (L) / 48
3.4 Column Packing Characteristics / 50
3.4.1 Support Type / 50
3.4.2 Particle Size (d_p) / 51
3.4.3 Surface Area and Pore Size (d_{pore}) / 51
3.4.4 Bonding Chemistries / 52
3.5 Modern HPLC Column Trends / 54
3.5.1 Silica Support Material / 54
3.5.2 Hybrid Particles / 55

3.5.3 Novel Bonding Chemistries / 58
3.5.3.1 Bonded Phases for Retention of Polar Analytes / 59
3.5.3.2 Charged Surface Hybrid (CSH) / 61
3.5.4 Shorter and Narrower Columns Packed with Small Particles / 61
3.5.4.1 Fast LC / 61
3.5.4.2 UHPLC / 62
3.5.5 Micro-LC and Nano-LC / 62
3.5.6 Monoliths / 64
3.5.7 Superficially Porous Particles (SPP) / 65
3.5.7.1 Kinetic Plots Demonstrating the Superiority of SPP / 66
3.5.8 Micropillar Array Chromatography (μPAC) / 67
3.6 Guard Columns / 69
3.7 Specialty Columns / 69
3.7.1 Bioseparations Columns / 69
3.7.2 Chiral Columns / 69
3.7.3 Supercritical Fluid Chromatography (SFC) Columns / 71
3.7.4 Hydrophilic Interaction Liquid Chromatography (HILIC) Columns / 72
3.7.5 Mixed-Mode Chromatography (MMC) Columns / 72
3.7.6 Application-Specific Columns / 73
3.8 RPC Column Selection Guides / 73
3.8.1 Some General Guidelines for Bonded Phase Selection / 75
3.9 Summary / 76
3.10 Quizzes / 76
3.10.1 Bonus Quiz / 78
References / 78

4 HPLC/UHPLC Instrumentation and Trends 81

4.1 Introduction / 81
4.1.1 Scope / 81
4.1.2 HPLC Systems and Modules / 81
4.1.3 Ultra-High-Pressure Liquid Chromatography (UHPLC) / 83
4.2 HPLC and UHPLC Solvent Delivery Systems / 83
4.2.1 High-Pressure and Low-Pressure Mixing Designs in Multisolvent Pumps / 85
4.2.2 System Dwell Volume / 86
4.2.2.1 Dwell Volumes of UHPLC Systems / 87
4.2.3 Trends / 88
4.3 Injectors and Autosamplers / 88
4.3.1 Operating Principles of Autosamplers / 88
4.3.2 Performance Characteristics and Trends / 89
4.4 Detectors / 91
4.5 UV/VIS Absorbance Detectors / 92
4.5.1 Operating Principles / 92
4.5.2 Performance Characteristics / 94
4.5.3 Trends in UV/Vis Absorbance Detectors / 94
4.6 Photodiode Array Detectors / 94
4.6.1 Operating Principles / 94

4.6.2 Trends in PDA Detectors / 95
4.7 Other Detectors / 95
4.7.1 Refractive Index Detector (RID) / 96
4.7.2 Evaporative Light Scattering Detector (ELSD) / 96
4.7.3 Charged Aerosol Detector (CAD) / 97
4.7.4 Conductivity Detector (CD) / 97
4.7.5 Fluorescence Detector (FLD) / 97
4.7.5.1 Postcolumn Reaction Technique / 98
4.7.6 Chemiluminescence Nitrogen Detector (CLND) / 98
4.7.7 Electrochemical Detector (ECD) / 99
4.7.8 Radiometric Detector / 99
4.8 Hyphenated and Specialized Systems / 99
4.8.1 LC/MS and LC/MS/MS / 99
4.8.2 LC/NMR / 100
4.8.3 Other Hyphenated Systems / 102
4.8.4 Supercritical Fluid Chromatography (SFC) / 102
4.8.5 Preparative LC and SFC / 102
4.8.6 Micro- and Nano-LC (Capillary LC) / 102
4.8.7 Multidimensional LC / 102
4.8.8 Lab-on-a-Chip / 104
4.8.9 Specialized Applications Systems / 104
4.8.9.1 Gel-Permeation Chromatography (GPC) / 104
4.8.9.2 Ion Chromatography (IC) / 105
4.8.9.3 Application-Specific Systems / 105
4.9 HPLC Accessories / 105
4.9.1 Solvent Degasser / 105
4.9.2 Column Oven / 105
4.9.3 Valves for Column and Mobile Phase Selection / 106
4.10 Chromatography Data Systems (CDS) / 106
4.10.1 User Interface and CDS Workflow / 107
4.11 Instrumental Bandwidth (IBW) / 108
4.11.1 How to Measure IBW / 109
4.11.2 IBW of UHPLC Systems / 110
4.12 Manufacturers and Equipment Selection / 111
4.13 Trends in HPLC and UHPLC Equipment / 111
4.14 Summary / 112
4.15 Quizzes / 112
4.15.1 Bonus Quiz / 114
References / 114

5 UHPLC: Perspectives, Performance, Practices, and Potential Issues 117

5.1 Introduction / 117
5.1.1 Scope / 117
5.1.2 Glossary and Abbreviations / 117
5.1.3 Historical Perspectives: What is UHPLC? / 118
5.2 Practical Concepts in UHPLC / 120
5.2.1 Rationale for Higher System Pressure / 120
5.2.2 Rationale for Low-Dispersion Systems / 121

5.2.3 Rationale for Low Dwell Volumes / 121
5.2.4 Other UHPLC Instrumental Characteristics / 122
5.3 Benefits Of UHPLC and Case Studies / 122
5.3.1 Benefit #1: Fast Separations with Good Resolution / 122
5.3.2 Benefit #2: High-Resolution Analysis of Complex Samples / 124
5.3.3 Benefit #3: Rapid HPLC Method Development / 124
5.3.4 Flexibility for Customizing Resolution / 129
5.3.5 Other Benefits of UHPLC / 130
5.3.5.1 Solvent Saving / 130
5.3.5.2 Higher Mass Sensitivity in UV Detection / 130
5.3.5.3 Higher Precision Performance for Retention Time and Peak Area / 130
5.3.5.4 UHPLC are Compatible with Other Approaches / 131
5.4 Potential Issues and How to Mitigate / 132
5.4.1 Safety Issues / 132
5.4.2 Viscous Heating / 133
5.4.3 Instrumental and Operating Nuances / 133
5.4.4 Injector Precision / 135
5.4.5 UV Detection Noise vs. Mixer Volumes / 135
5.4.6 Method Translation (Conversion) / 138
5.4.6.1 Running the Same HPLC Methods on HPLC and UHPLC / 138
5.4.6.2 Back Conversion of UHPLC Methods to HPLC Method Conditions / 139
5.4.6.3 Conversion of Existing HPLC Methods to Faster UHPLC Methods / 139
5.4.6.4 Method Validation Requirements After Method Translation / 139
5.5 How to Implement UHPLC and Practical Aspects / 139
5.5.1 How to Transition from HPLC to UHPLC / 139
5.5.2 End-Fittings / 140
5.5.3 A Summary of UHPLC System Performance Tradeoffs / 140
5.6 Myths in UHPLC / 142
5.7 Summary and Conclusions / 142
5.8 Quizzes / 142
5.8.1 Bonus Quiz / 144
References / 144

6 LC/MS: Fundamentals, Perspectives, and Applications 147
Christine Gu

6.1 Introduction / 147
6.1.1 Scope / 147
6.1.2 LC/MS Technology and Instrumentation / 147
6.1.3 Basic Terminologies and Concepts for MS / 148
6.1.4 Interfacing HPLC and MS / 150
6.2 LC/MS Instrumentation / 150
6.2.1 Ion Sources / 150
6.2.2 Fragmentation / 152
6.2.3 Mass Analyzers / 153

6.2.4 Detectors / 155
6.3 Small-Molecules Drug Research and Development / 157
6.3.1 Mass Measurement and Elemental Composition Determination / 157
6.3.2 Structural Elucidation / 159
6.3.3 Trace Quantitation / 162
6.4 Emerging Biopharmaceutical Applications / 164
6.4.1 Intact Mass Measurement of Proteins / 166
6.4.2 Structural Characterization of Proteins (Bottom-Up and Top-Down Approaches) / 166
6.4.3 Peptide Quantitation / 170
6.5 Environmental, Food Safety, Clinical, Toxicology, and "Omics" Applications / 171
6.6 Future Perspectives / 171
6.7 Quizzes / 172
6.7.1 Bonus Quiz / 174
References / 174

7 HPLC/UHPLC Operation Guide 177

7.1 Scope / 177
7.2 Safety and Environmental Concerns / 177
7.2.1 Safety Concerns / 177
7.2.2 Environmental Concerns / 179
7.3 Mobile Phase and Sample Preparation / 180
7.3.1 Mobile Phase Premixing / 180
7.3.2 Mobile Phase Additives and Buffers / 180
7.3.3 Filtration / 180
7.3.4 Degassing / 181
7.3.5 Samples, Diluents, and Sample Preparation / 181
7.4 Best Practices in HPLC/UHPLC System Operation / 182
7.4.1 Pump Operation / 182
7.4.2 HPLC Column Use, Precaution, Connection, and Maintenance / 183
7.4.2.1 Column Use / 183
7.4.2.2 Column Precautions / 183
7.4.2.3 Column Connections / 183
7.4.2.4 Column Maintenance and Regeneration / 184
7.4.3 Autosampler Operation / 184
7.4.4 Column Oven and Switching Valve / 186
7.4.5 UV/Vis Detector Operation / 186
7.4.6 HPLC System Shutdown / 187
7.4.7 Guidelines for Increasing HPLC Precision / 187
7.4.7.1 Guidelines for Improving Retention Time Precision / 187
7.4.7.2 Guidelines for Improving Peak Area Precision / 188
7.5 From Chromatograms to Reports / 189
7.5.1 Qualitative Analysis Strategies / 192
7.5.2 Quantitation Analysis Strategies / 192
7.6 Summary of HPLC Operation / 193

7.7 Guides on Performing Trace Analysis / 193
7.8 Summary / 195
7.9 Quizzes / 195
7.9.1 Bonus Quiz / 196
References / 196

8 HPLC/UHPLC Maintenance and Troubleshooting 199

8.1 Scope / 199
8.2 HPLC System Maintenance / 199
8.2.1 HPLC Pump / 200
8.2.2 UV/Vis Absorbance or Photodiode Array Detectors (PDA) / 202
8.2.3 Injector and Autosampler / 204
8.3 HPLC Troubleshooting / 204
8.3.1 General Problem Diagnostic and Troubleshooting Guide / 205
8.3.2 Common HPLC Problems / 206
8.3.2.1 Pressure Problems and Causes / 207
8.3.2.2 Baseline Problems (Chromatogram) / 207
8.3.2.3 Peak Problems (Chromatogram) / 210
8.3.2.4 Data Performance Problems / 213
8.4 Troubleshooting Case Studies / 213
8.4.1 Case Study 1: Reducing Baseline Shift and Noise for Gradient Analysis / 213
8.4.2 Case Study 2: Poor Peak Area Precision / 214
8.4.3 Case Study 3: Poor Assay Accuracy Data / 215
8.4.4 Case Study 4: Equipment Malfunctioning and Problems with Blank / 216
8.5 Summary and Conclusion / 217
8.6 Quizzes / 218
8.6.1 Bonus Quiz / 219
References / 219

9 Pharmaceutical Analysis 221

9.1 Introduction / 221
9.1.1 Scope / 221
9.1.2 Glossary and Abbreviations / 221
9.2 Overview of Drug Development Process / 222
9.3 Sample Preparation Perspectives / 224
9.4 HPLC, SFC, and HPLC/MS in Drug Discovery / 224
9.5 HPLC Testing Methodologies for DS and DP / 225
9.5.1 Identification Test (DS, DP) / 227
9.5.2 ASSAY (Rough Potency and Performance Testing, DP) / 227
9.5.2.1 Testing for Uniformity of Dosage Units / 227
9.5.2.2 Dissolution Testing / 228
9.5.3 Stability-Indicating Assay (Potency and Purity Testing of DS and DP) / 230
9.5.3.1 Trends in Stability-Indicating Methods / 230
9.5.3.2 Potency Determination (DS, DP) / 231
9.5.3.3 Qualification of Reference Standard for the API / 232

9.5.3.4 Quantitation of Impurities and Degradation Products (DS, DP) / 233
9.5.3.5 Case Study of an HPLC Method of an API with Multiple Chiral Centers / 234
9.5.3.6 Control of Chemical and Chiral Purities of Starting Materials for Multichiral APIs / 236
9.5.3.7 DP with Multiple APIs or Natural Products / 237
9.5.3.8 Stability Studies / 237
9.5.4 Assay of Preservatives / 238
9.5.5 Assay of Pharmaceutical Counterions / 238
9.5.6 Assay of Potential Genotoxic Impurities (PGI) / 239
9.6 Cleaning Verification / 239
9.7 Bioanalytical Testing / 240
9.8 Summary / 242
9.9 Quizzes / 242
9.9.1 Bonus Quiz / 243
References / 243

10 HPLC Method Development 245

10.1 Introduction / 245
10.1.1 Scope / 245
10.1.2 Considerations Before Method Development / 245
10.1.3 HPLC Method Development Trends in Pharmaceutical Analysis / 246
10.2 A Five-Step Strategy for Traditional HPLC Method Development / 246
10.2.1 STEP 1: Defining Method Types and Goals / 246
10.2.1.1 Method Goals and Acceptance Criteria for Quantitative Assays / 247
10.2.2 STEP 2: Gathering Sample and Analyte Information / 248
10.2.3 STEP 3: Initial HPLC Method Development / 248
10.2.3.1 Initial Detector Selection / 249
10.2.3.2 Selection of Chromatographic Mode / 250
10.2.3.3 Selecting Samples / 250
10.2.3.4 Initial Selection of HPLC Column and Mobile Phase / 251
10.2.3.5 Generating the First Chromatogram / 251
10.2.3.6 Case Study: Initial Purity Method Development of an NCE Using a Broad Gradient / 251
10.2.4 STEP 4: Method Fine-Tuning and Optimization / 253
10.2.4.1 Mobile Phase Parameters (%B, pH, Buffer, Solvent Type) / 255
10.2.4.2 Operating Parameters (F, T, $\Delta\Phi$, t_G, Gradient Segment) / 255
10.2.4.3 Column Parameters (Bonded Phase Type, L, d_p, d_c) / 255
10.2.4.4 Detector Setting and Sample Amount / 256
10.2.5 STEP 5: Method Prequalification / 256
10.2.6 Summary of Method Development Steps / 257
10.2.7 Phase-Appropriate Method Development and Validation / 257
10.2.8 Method Development Software Tools / 258

10.3 Case Studies / 258
10.3.1 A Phase-0 Drug Substance Method for an NCE / 259
10.3.2 Stability-Indicating Method Development for an NCE Using DryLab / 260
10.3.3 Stability-Indicating Method for a Combination Drug Product with Two APIs / 262
10.3.4 Automated Method Development System Employing Fusion QbD Software / 265
10.4 A Three-Pronged Template Approach for Rapid HPLC Method Development / 268
10.4.1 Template #1: Fast LC Isocratic Potency or Performance Methods / 269
10.4.2 Template #2: Generic Broad Gradient Methods / 270
10.4.3 Temple #3 Multisegment Gradient Methods for NCEs / 271
10.4.4 Summary of the Three-Pronged Approach / 272
10.5 A Universal Generic Method for Pharmaceutical Analysis / 272
10.5.1 Rationales for the Generic Method Parameters / 272
10.5.2 Adjustment of the Generic Method for Stability-Indicating Assays / 273
10.5.3 Summary of the Universal Generic Method Approach / 275
10.6 Comments on Other HPLC Modes / 276
10.7 Summary and Conclusions / 276
10.8 Quizzes / 277
10.8.1 Bonus Quiz / 278
References / 278

11 Regulations, HPLC System Qualification, Method Validation, and Transfer 281

11.1 Introduction / 281
11.1.1 Scope / 281
11.1.2 Glossary and Abbreviations / 281
11.2 Regulatory Environment in the Pharmaceutical Industry / 281
11.2.1 Regulations / 283
11.2.1.1 Good Manufacturing Practice (GMP) / 283
11.2.1.2 International Council for Harmonization (ICH) Guidelines / 283
11.2.1.3 Good Laboratory Practice (GLP) / 284
11.2.2 The Role of the United States Food and Drug Administration (U.S. FDA) / 284
11.2.3 The United States Pharmacopeia (USP) / 284
11.3 HPLC System Qualification / 285
11.3.1 Design Qualification (DQ) / 285
11.3.2 Installation Qualification (IQ) / 285
11.3.3 Operational Qualification (OQ) / 287
11.3.4 Performance Qualification (PQ) / 287
11.3.5 System Qualification Documentation / 287
11.3.6 System Calibration / 287
11.3.7 System Suitability Testing (SST) / 289

11.4 Method Validation / 290
11.4.1 Data Required for Method Validation / 291
11.4.1.1 Specificity / 291
11.4.1.2 Linearity and Range / 292
11.4.1.3 Accuracy / 294
11.4.1.4 Precision: Repeatability, Reproducibility / 294
11.4.1.5 Sensitivity: Detection Limit and Quantitation Limit / 295
11.4.1.6 Robustness / 296
11.4.2 Case Studies and Summary Data on Method Validation / 296
11.5 Method Transfer / 298
11.6 Regulatory Filings / 298
11.7 Cost-Effective Regulatory Compliance Strategies / 298
11.7.1 Regulatory Compliance in Other Industries / 301
11.8 Summary and Conclusions / 302
11.9 Quizzes / 302
11.9.1 Bonus Quiz / 303
References / 303

12 HPLC and UHPLC for Biopharmaceutical Analysis 305
Jennifer Rea and Taylor Zhang

12.1 Introduction / 305
12.2 Size-Exclusion Chromatography (SEC) / 308
12.2.1 SEC Introduction / 308
12.2.2 SEC Theory and Fundamentals / 308
12.2.3 SEC Method Conditions / 309
12.2.4 SEC Applications / 311
12.3 Ion-Exchange Chromatography (IEC) / 312
12.3.1 IEC Introduction / 312
12.3.2 IEC Theory and Fundamentals / 313
12.3.3 IEC Method Conditions / 313
12.3.4 IEC Applications / 314
12.4 Affinity Chromatography / 314
12.4.1 Affinity Chromatography Introduction / 314
12.4.2 Affinity Chromatography Theory and Fundamentals / 315
12.4.3 Affinity Chromatography Method Conditions / 315
12.4.4 Affinity Chromatography Applications / 316
12.5 Hydrophilic Interaction Liquid Chromatography (HILIC) / 317
12.5.1 HILIC Introduction / 317
12.5.2 HILIC Theory and Fundamentals / 317
12.5.3 HILIC Method Conditions / 318
12.5.4 HILIC Applications / 318
12.6 Reversed-Phase Chromatography (RPC) / 320
12.6.1 RPC Introduction / 320
12.6.2 RPC Theory and Fundamentals / 320
12.6.3 RPC Method Conditions / 321
12.6.4 RPC Applications / 321
12.7 Hydrophobic Interaction Chromatography (HIC) / 322
12.7.1 HIC Introduction / 322

12.7.2 HIC Theory and Fundamentals / 322
12.7.3 HIC Method Conditions / 323
12.7.4 HIC Applications / 324
12.8 Mixed-Mode Chromatography (MMC) / 324
12.8.1 MMC Introduction / 324
12.8.2 MMC Theory and Fundamentals / 325
12.8.3 MMC Method Conditions / 325
12.8.4 MMC Applications / 325
12.9 Multidimensional Liquid Chromatography / 326
12.9.1 Multidimensional LC Introduction / 326
12.9.2 Multidimensional LC Theory and Fundamentals / 326
12.9.3 Multidimensional LC Method Conditions / 327
12.9.4 Multidimensional LC Applications / 327
12.10 Summary / 328
12.11 Quizzes / 328
References / 329

13 HPLC Applications in Food, Environmental, Chemical, and Life Sciences Analysis 335

13.1 Introduction / 335
13.1.1 Scope / 335
13.2 Food Applications / 335
13.2.1 Natural Food Components / 336
13.2.1.1 Sugars / 336
13.2.1.2 Fats, Oils, and Triglycerides / 337
13.2.1.3 Free Fatty Acids and Organic Acids / 338
13.2.1.4 Proteins and Amino Acids / 340
13.2.2 Food Additives / 341
13.2.2.1 Flavors: A Case Study on HPLC Analysis of Capsaicins / 345
13.2.3 Contaminants / 346
13.2.3.1 Mycotoxins / 347
13.2.3.2 Antimicrobial Additives / 348
13.2.3.3 Pesticide Residues / 348
13.3 Environmental Applications / 349
13.3.1 Listing of U.S. EPA Test Methods Using HPLC / 349
13.3.2 Pesticides Analysis / 349
13.3.2.1 Carbamates and Glyphosate / 350
13.3.3 Polynuclear Aromatic Hydrocarbons (PAH) / 351
13.3.3.1 Case Study: Quick Turnaround Analysis of PAHs in Multimedia Samples / 351
13.3.4 HPLC Analysis of Carbonyl Compounds (Aldehydes and Ketone) / 352
13.4 Chemical Industry, GPC, and Plastics Applications / 352
13.4.1 Gel-Permeation Chromatography (GPC) and Analysis of Plastics Additives / 352
13.5 Ion Chromatography (IC) / 356
13.6 Life Sciences Applications / 356

13.6.1 Proteins, Peptides, and Amino Acids / 357
13.6.2 Bases, Nucleosides, Nucleotides, Oligonucleotides, and Nucleic Acids / 363
13.6.3 Bioscience Research in Proteomics, Metabolomics, Glycomics and Clinical Diagnostics / 363
13.7 Summary / 366
13.8 Quizzes / 366
13.8.1 Bonus Questions / 368
References / 368

Keys to Quizzes **371**

Index **373**